PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : B32B 5/16 | A1

(11) International Publication Number: WO 99/52708

(43) International Publication Date: 21 October 1999 (21.10.99)

(21) International Application Number: PCT/US99/07993

(22) International Filing Date: 13 April 1999 (13.04.99)

(30) Priority Data:
60/081,487 13 April 1998 (13.04.98) US

(71) Applicant: LUMINEX CORPORATION [US/US]; 12212 Technology Boulevard, Austin, TX 78727-6115 (US).

(72) Inventor: CHANDLER, Van, S.; 7300 Valburn Drive, Austin, TX 78731 (US).

(74) Agents: VILLACORTA, Gilberto, M. et al.; Pepper Hamilton LLP, 600 Fourteenth Street, N.W., Washington, DC 20005-2004 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, ~~UG~~, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
With amended claims.

(54) Title: LIQUID LABELING WITH FLUORESCENT MICROPARTICLES

(57) Abstract

Methods of labeling liquids, solids, aerosols, and gases with fluorescent microparticles and/or with microparticles encoded with at least one other discriminator. Labeling protocols are described for such uses as adding at least one date code (for example, the expiration date of a perishable liquid), encoding routing information for one or more liquids in a pipeline having a plurality of branch points, tracing the flow of toxic or other substances into groundwater, tracing liquid flow from a point source into groundwater, tracing groundwater flow, adding a donor Social Security Number, adding a donor name, tracing flow of a liquid or a gas in a living system, adding unique identification to each of a plurality of liquid, solid or gaseous samples, encoding routing information to each of a plurality of liquid samples in a multiplexed analytical system, encoding routing information to each of a plurality of liquid reagents in a multiplexed analytical system, and combinations thereof. In one embodiment, two different fluorescent dye labels are bound to microparticles at eight independent, different concentrations each to create an 8 x 8 = 64 array of distinct microparticle sets suitable for labeling liquids, solids, and gases. When three discriminators are used at 8 levels each, an 8 x 8 x 8 = 512 array of distinct microparticle sets is created. Discriminators include such parameters as particle physical property, particle concentration, spectral property, dye type, dye concentration, label type, and combinations thereof; where particle physical property includes such attributes as particle size, particle shape, particle hydrophobicity, particle hydrophilicity, particle density, and combinations thereof; where spectral property includes such attributes as dye fluroescence absorption, dye fluorescence emission, dye absorption, dye fluorescence polarization, dye fluorescence lifetime, and combinations thereof; and where label type includes such attributes as magnetic property, reactive group, nuclear magnetic resonance, electron spin resonance, positron emission, radioactive property, fluorescence polarization, fluorescence lifetime, and combinations thereof. Kits are devised to permit users to create a large variety of sets of particles for identification purposes in liquids, solids, and gases, and microprocessor controlled, automatic pipetting of kit microparticles are also encompassed by the instant invention.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

LIQUID LABELING WITH FLUORESCENT MICROPARTICLES

1.0 Cross Reference to Related Applications

This Application claims the priority date of Provisional U.S. Application Serial No. 60/081,487, filed April 13, 1998, the disclosure of which is herein incorporated by reference in its entirety.

2.0 Field of the Invention

The present invention relates generally to a method of labeling liquids. In particular, the present invention relates to a method of labeling liquids with particles, and to flow cytometric amenable methods of encoding such labels in liquids. Such particles and methods of their use also have applications in labeling solids and gases.

3.0 Background of the Invention

Knowing the correct identity of different liquids is critical in a variety of contexts. For example, the absolute identity of different grades of aviation fuel is required for optimum performance and safety in airplanes and other aircraft. Typically, different grades of aviation fuel are identified according to color in the visual spectrum, based on dyes added exogenously.

Fluids required by automobiles, other vehicles, motorized tools and equipment in general provide additional examples of the need for correct identification of liquids. For such motorized mechanical devices, fluids therefor are colored according to the mechanical compartment for which use is intended. Such color coding clearly is designed to prevent, or at least minimize the possibility of, damage or even total destruction of the mechanical device that would result from introduction of the incorrect fluid into a given mechanical compartment. Thus, the color of the antifreeze solution is different from that of the brake fluid, etc.

In addition, as a part of a quality assurance or quality control program, there often is a need to verify the identities of various biological and other fluids that are undergoing analysis. This is particularly true in situations where integrity and identity of a sample is critical, for example, in legal situations such as court ordered testing. Examples of biological fluids that could benefit from labeling include separated or unfiltered biological fluids such as urine, cerebrospinal fluid, blood, serum, plasma, lymph fluids, tissue homogenates, interstitial fluid, cell extracts, mucus, saliva, sputum, stool, semen, vaginal secretions, lacrimal fluid, physiological secretions, or other similar

fluids. Examples can also include samples obtained from environmental sources, such as soil, water, or air; or from industrial sources, such as those taken from a waste stream, a water source, a supply line, or a production lot. Industrial sources can include such fluids as fermentation media, for example, from a biological reactor or food fermentation process, or fluids generated from foodstuffs, such as from meat, grain, produce, or dairy products.

Liquids are notoriously difficult to label and thus identify. The use of visible spectrum color indicators for fluid identification suffer from at least two inherent disadvantages: 1) a limited range of separately identified liquids due to the limited range of colors that are readily distinct to the human eye, and 2) ease of alteration and/or sabotage of the liquid for which the easy, visual based identification is sought. In addition, a significant portion of the human population suffers from color blindness, thereby increasing problems of identification related to the use of visible spectrum color indicators. This difficulty is further compounded because clinically distinct forms of color blindness have different wavelength ranges over which color perception is absent or impaired.

In addition to the perception of color, viscosity, odor, taste, and "feel" can also provide useful identification parameters. However, not only are these alternate sensory modes of identification typically impractical, or even dangerous to perform, they generally give ambiguous and inconclusive results.

A need therefore exists for a means to label liquids which is precise, tamper proof, reproducible, and easily read.

Fluorescent dyes and lasers have been used for a number of years to label and detect cells, biochemical interactions, and uniform latex particles. For such uses, laser techniques often are utilized to determine the presence or absence of a dyed constituent, and/or concentrations thereof. Fluorescent dyes have been applied to such uses as: (1) detecting the presence (and quantity) or absence of cells and/or specific types of cells in a fluid sample; (2) gauging cell vitality; (3) estimating pH and/or changes in pH in cellular compartments; (4) determining intracellular pH measurement in general; (5) identifying acidic vesicles in cells; (6) identifying DNA; (7) identifying "GC" rich or "AT" rich regions of DNA; (8) identifying cellular surface markers; (9) quantifying analytes by ELISA plate assays; (10) estimating blood flow; (11) labeling proteins for gel electrophoresis analysis; (12) labeling cellular cytoskeleton or other intracellular proteins; (13) identifying hydrophobic and hydrophilic sites on proteins and membranes; (14) measuring total amounts of protein in a sample; (15) measuring intra-molecular distances by energy transfer; (16) performing optical sectioning with confocal microscopy; (17) measuring and studying various molecular and physical chemical properties and biochemical parameters, including enzyme-substrate interactions, as well as interactions between enzymes and various modulators (activators, inhibitors,

cofactors, etc.) or other molecules, including interactions with homologous and heterologous protein subunits, and with antibodies; and (18) detecting the presence of, absence of, and/or number of, uniform dyed particles in a liquid sample.

According to the practice of earlier technologies, particles often have been labeled with a single fluorescent dye. In general, these particles are made by a copolymerization process, wherein monomers, such as unsaturated aldehyde or acrylate, are allowed to polymerize in the presence of a fluorescent dye, such as fluorescein isothiocyanate or "FITC," in the reaction mixture. For example, see U.S. Patent Nos. 4,267,234 to Rembaum, 4,267,235 to Rembaum, et al., 4,552,812 to Margel, et al. and 4,677,138 to Margel.

The use of two or more dyes of varying proportions would increase the permutation number of unique combinations of dyes in or on a single particle. Particles having varying combinations of unique characteristics, such as differences in emission wavelengths and/or fluorescence intensities, could be extremely useful for labeling liquids.

However, several problems arise when more than one dye is absorbed into or onto a particle. First, the close proximity of embedded dye molecules gives rise to significant amounts of fluorescent energy transfer (for example, Stokes shifts). This energy transfer leads to fluorescent emissions that are inconsistent with relative dye concentrations and their original emission patterns.

Second, difficulties arise when the dyes have differing solubilities in the solvent used to incorporate the dye in or on the particles. Typically, all dyes must be absorbed simultaneously; otherwise, in practice, possible dye ratios are restricted by solvent properties.

A third problem that has been encountered when multiple dyes are embedded in particles is the change in dye spectra. Specifically, it has been noted that, when the particle is composed of crosslinked polystyrene, a significant broadening of the fluorescent emission peak occurs. This can result in an overlapping of the emission spectra of neighboring dyes.

A need therefore exists for multicolored, fluorescent particles comprising a plurality of defined sets of stained multicolored microparticles, and/or a plurality of defined sets of dyed nanoparticles attached to a carrier microparticle, which can be used for labeling liquids. It would also be desirable to use such microparticles or carrier microparticles in conjunction with other labeling elements ("discriminators"), such as radioactive property, nuclear magnetic resonance, electron spin resonance, chemical reactive group, dye fluorescence absorption, dye fluorescence emission, fluorescence polarization, fluorescence lifetime, positron emission, concentration of particles, particle hydrophobicity, particle hydrophilicity, particle density, particle size, particle shape, dye concentration in or on particles, and dye type. Applications of this invention include, but are not limited to: (1) adding date codes to perishable or other liquids; (2) adding routing

information to liquids in a pipeline; (3) tracing toxic substances in groundwater; (4) adding donor's Social Security Number to blood units; (5) tracing absorption and/or secretion and excretion patterns of fluids into, out of, or within plant and animal tissues; (6) adding patient identification to clinical samples; (7) adding identifiers to clinical reagents; and (8) adding routing information to liquids in a process stream, for example, a plurality of liquid samples from one or more patients or other sources of samples, where the liquid samples are each routed to an analytical machine for a specific assay, or to an analytical machine for a panel of assays.

4.0 Summary of the Invention

It is therefore an object of the present invention to provide methods, instrumentation, and products for precisely and unambiguously labeling fluids (liquids, aerosols, and gases), and solids.

It is a further object of the present invention to provide methods, instrumentation, and products for precisely and unambiguously reading the labels within the fluids (liquids, aerosols, and gases), and solids.

The present invention uses precisely dyed microparticles and/or nanoparticles attached to carrier microparticles, and also may utilize a variety of other discriminators. In a preferred embodiment, the particles are made of polystyrene, crosslinked with 5 percent divinyl-benzene, and have a diameter of about 5.5 microns. The particles are impregnated with three different fluorescent dyes in eight different concentrations of each of the dyes, so as to create 512 subsets of labeled particles. The subset classification of each particle is identified by fluorescence measurement using appropriate instrumentation, such as a flow cytometer. Depending upon the needs of a particular application, the discriminators can be combined in a variety of ways to achieve the required number of unique labels. A first embodiment uses precisely dyed microparticles (core microparticles). In an alternative embodiment, precisely dyed nanoparticles are attached to carrier microparticles. Other embodiments incorporate such discriminators as radioactive property, nuclear magnetic resonance, electron spin resonance, chemical reactive group, dye fluorescence absorption, dye fluorescence emission, fluorescence polarization, fluorescence lifetime, positron emission, concentration of particles, particle hydrophobicity, particle hydrophilicity, particle density, particle size, particle shape, dye concentration in or on particles, and dye type. Thus, use of microparticles according to the present invention functions as an identifying means analogous to a bar code.

Multiplexed diagnostic instruments, such as those in the current art (including flow cytometers), can be programmed to recognize each specific permutation or particle set, look up the parameters in a database, and identify the sample's origin/destination and/or perform the appropriate analysis or routing function.

The present invention provides a method of labeling a gaseous, liquid, or solid material, including combinations thereof, to permit the subsequent identification of the labeled gaseous, liquid, or solid material, which comprises adding to a gaseous, liquid, or solid material, whose subsequent identification is desired, a sufficient quantity of particles belonging to one or more distinguishable sets of particles, to provide a labeled gaseous, liquid, or solid material that subsequently can be identified. In a particular embodiment of the invention, the method makes use of particles in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set. In particular, the one or more discriminators is selected from a physical, chemical, biological, magnetic, or spectral property of the particle or combinations thereof. Specific properties are described further in the detailed description of the preferred embodiments of the invention and others may be apparent to those of ordinary skill in the art.

In the present method, for example, a sufficient quantity of a combination of particles belonging to two or more distinguishable sets of particles is added to the gaseous, liquid, or solid material, whose subsequent identification is desired.

The present invention also provides a method of identifying a gaseous, liquid, or solid material, including combinations thereof, which has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles. This method comprises: (a) obtaining a sample comprising a gaseous, liquid, or solid material that has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles; (b) analyzing the particles found in the sample to determine which of one or more distinguishable sets of particles such particles belong to; and (c) comparing the results of the analyzing step with a key which establishes the identity of the gaseous, liquid, or solid material from which the sample was taken.

In various embodiments, the present invention makes possible the manufacture, use and sale of labeled materials which comprise (i) a gaseous, liquid, or solid material, including combinations thereof, and (ii) a sufficient quantity of particles that can be shown to belong to one or more distinguishable sets of particles. In a preferred embodiment the labeled gaseous, liquid or solid material makes use of particles in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set. Kits for labeling liquids, solids, aerosols, and gases comprise particles from a plurality of distinct particle sets, and a dispenser.

6.0 Description of the Specific Embodiments

One method of practice of the present invention involves the use of precisely dyed core microparticles. An alternative method of practice entails the use of carrier microparticles to which nanoparticles comprising a plurality of dyes with spectrally distinct fluorescence emission spectra are attached, preferably covalently. The following summarizes exemplary but non-limiting embodiments for the production of such labeled particles suitable for use in practice of the present invention, as well as how to use such particles to label liquids, and subsequently identify such labels, each of which may be used to uniquely identify a specific liquid. As used herein: (1) the terms nanospheres and nanoparticles have equivalent meanings; (2) in the context of incorporating labels (fluorescent and otherwise) into or onto particles, the terms attach, attachment, bound, coupled, etc., are used broadly to include covalent and non-covalent incorporation of chemicals and dyes, particles, etc., as well as entrapment of such entities within the matrix of particles ("matrix entrapment"), for example, subsequent to the swelling of a particle; (3) the terms microparticles, microspheres, or microbeads bear equivalent meanings; (4) particles refers to either microparticles or nanoparticles; (5) where nanoparticles are attached to microparticles, the microparticles are referred to as carrier microparticles; and (6) where microparticles are precisely dyed, the microparticles are referred to as core microparticles.

6.1 Particles

In one embodiment, nanoparticles are coupled to carrier microparticles to form sets of distinct particles. Basically, fluorescently distinguishable particle sets are obtained through variation of the amount or type of dye in or on each nanoparticle. Then, the desired number of dye categories of nanoparticles are attached to the surface of each carrier microparticle. Ratios of differently dyed nanoparticles attached to the surfaces of the carrier microparticles are selected according to the particular need, and in accord with the encoding or identification criteria developed therefor. These ratios reflect differences in the dyes used, and in their respective quantities attached to each particle.

Similarly, and in an alternative embodiment, fluorescently distinguishable microparticle sets are obtained through variation of the amount or type of dye in or on each microparticle, and no nanoparticles are utilized. The encoding or identification criteria can be identical to those used with nanoparticles.

Nanoparticles used in this invention are commercially available in sizes ranging from about 5 to 10 nanometers (nm) to more than 1,000 nm in diameter. Optimal diameters typically will fall within the range from about 10 to about 1,000 nm, preferably from about 200 to about 500 nm. Nanoparticles as small as about 2-3 nm can also be prepared.

Microparticles that are used as carrier particles, to which the nanoparticles are attached, typically range in diameter from about 0.1 to about 100 micrometers (μm). Even though a microparticle for practicing the present invention can be of any size, the preferred diameter usually will range from about 0.1 to about 50 μm, more preferably from about 1 to about 20 μm, and even more preferably from about 2 to about 12 μm. The particles preferably will exhibit a high degree of uniformity with respect to size, or to a set of discretely different size classes such that 1) the differences can be determined by size-dependent properties, such as light scattering, and 2) the high degree of uniformity within each class size is sufficient to permit the use of particle size as a discriminator.

Microparticles used in this invention as core particles have a diameter of less than one millimeter, and preferably in the range of about 0.1 to 1,000 micrometers (μm). Even though the microparticle can be of any size, the preferred size is 1-100 μm, more preferably 2-50 μm, more preferably 3-25 μm, and even more preferably about 5-12 μm.

Particles are made of any approximately regularly shaped material. Often, the preferred shape will be approximately spherical; however, particles of any other shape can be employed since, for most uses, this parameter will be immaterial to practice of the instant invention. However, the shape of a class of particles also can serve as an additional discriminator, which can be detected by a flow cytometric method, for example, by a high-resolution slit-scanning method that measures forward and side light scattering parameters.

Usually the nanoparticles and the microparticles are made of the same material, such as polystyrene or latex. However, other polymeric materials are acceptable, including polymers selected from chemical groups such as carbohydrate-based polymers, polyaliphatic alcohols, poly(vinyl) polymers, polyacrylic acids, polyorganic acids, polyamino acids, co-polymers, block co-polymers, tert-polymers, polyethers, naturally occurring polymers, polyimids, surfactants, polyesters, branched polymers, cyclo-polymers, polyaldehydes, and mixtures thereof. More specifically, brominated polystyrene, polyacrylic acid, polyacrylonitrile, polyamide, polyacrylamide, polyacrolein, polybutadiene, polycaprolactone, polyester, polyethylene, polyethylene terephthalate, polydimethylsiloxane, polyisoprene, polyurethane, polyvinylacetate, polyvinylchloride, polyvinylpyridine, polyvinylbenzylchloride, polyvinyltoluene, polyvinylidene chloride, polydivinylbenzene, polymethylmethacrylate, polylactide, polyglycolide, poly(lactide-co-glycolide), polyanhydride, polyorthoester, polyphosphazene, or combinations thereof, are preferable. Representative combination polymers of which the polymeric particles can be composed include, for example, poly-(styrene-co-vinylbenzyl chloride-co-acrylic acid) (in approximately an 85:10:5 molar ratio), poly(styrene-co-acrylic acid) (in approximately a 99:1 molar ratio),

poly(styrene-co-methacrylic acid) (in approximately a 90:10 molar ratio), poly(styrene-co-acrylic acid-co-m&p-divinyl-benzene) (in approximately an 89:10:1 molar ratio), poly-(styrene-co-2-carboxyethyl acrylate) (in approximately a 90:10 molar ratio), poly(methyl methacrylate-co-acrylic acid) (in approximately a 70:30 molar ratio) and poly(styrene-co-butyl acrylate-co-methacrylic acid)(in approximately a 45:45:10 weight ratio). Other exemplary polymers that can be used for the formation of microparticles include homopolymers and copolymers of lactic acid and glycolic acid (PLGA) (for example, as described in U.S. Patent Nos. 5,213,812 to Ruiz; 5,417,986 to Reid, et al.; 4,530,840, 4,897,268, 5,075,109, and 5,360,610 to Tice, et al.; 5,102,872 to Singh, et al.; 5,384,133 to Boyes, et al.; and European Patent Application Publication Number 248,531 to Southern Research Institute); block copolymers, such as tetronic 908 and poloxamer 407 (for example, as described in U.S. Patent No. 4,904,479 to Illum); and polyphosphazenes (for example, as described in U.S. Patent No. 5,149,543 to Cohen, et al.). Particles formed from synthetic polymers (such as polystyrene, polyacrylamide, polyacrylate, or latex) are commercially available from numerous sources, such as Bio-Rad Laboratories (Richmond, Calif.) and LKB Produkter (Stockholm, Sweden). Particles formed from natural macromolecules and particles, such as agarose, crosslinked agarose, globulin, deoxyribose nucleic acid, and liposomes, are commercially available from a variety of sources, such as Bio-Rad Laboratories, Pharmacia (Piscataway, N.J.), and IBF (France). Particles formed from copolymers of polyacrylamide and agarose are commercially available from various sources, including IBF and Pharmacia. Magnetic particles are commercially available from such sources as Dynal Inc. (Great Neck, N.Y.). Other materials, such as carbohydrates (e.g., carboxymethyl cellulose, and hydroxyethyl cellulose), proteinaceous polymers, polypeptides, eukaryotic and procaryotic cells, viruses, lipids, metals, resins, rubbers, silicas, and silicones (e.g., polydimethyldiphenyl siloxane, glass, ceramic, and the like), also can be utilized, depending on the specific use therefor.

Nanoparticles are preferably made of the same material as the microparticles. However, if required or advantageous, they can be made of different material.

The particles typically will also contain approximately 1% to approximately 50% by weight of one or more cross-linking agents, such as divinyl benzene, ethylene glycol dimethacrylate, trimethylol propane trimethacrylate, or N,N'methylene-bis-acrylamide, or other functionally equivalent agent(s) known in the art. Crosslinking of a carbohydrate polymer, such as hydroxypropyl cellulose, can be achieved with such agents as adipic acid, sebacic acid, succinic acid, citric acid, 1,2,3,4-butanetetracarboxylic acid, or 1,10-decanedicarboxylic acid. In a preferred embodiment, microparticles and nanoparticles are made of polystyrene, and contain about 1% to about 50% by weight of divinyl benzene.

The particles also can have additional surface functional groups to facilitate attachment to each other or to yet another entity, examples including carboxylates, esters, alcohols, carbamides, aldehydes, amines, sulfur oxides, nitrogen oxides, and halides. For many practical applications, it is critical that the particles have surface carboxyl groups available for attachment of a reactive amine- or sulfhydryl-containing compound, for example. Such groups are preferably added to the particles by incorporating monomers containing such groups into the polymers. These monomers can include, but are not limited to, acrylic acid, methacrylic acid, itaconic acid, and the like. Alternatively, they can be added to the particles by further chemical reaction of a polymer having other precursor reactive groups, which then can be converted to carboxyl groups, for example, by hydrolysis of anhydrides, such as maleic anhydride, or by oxidation of surface methylol or aldehyde end groups. Other compounds, such as diamines, dihydrazides, mercaptoalkylamines and dimercaptans, can be attached to particles as linking moieties for later attachment of drugs, enzymes or other reactive species, including nanoparticles. Although the preferred attaching method is by covalent linkage, non-covalent binding methods, such as adsorption, can also be used.

6.2 Dyes

A dye is selected to give a detectable response that is different from that of other dyes and /or discriminators. Any fluorescence detection system (including visual inspection) can be used to detect differences in spectral properties between dyes, with differing levels of sensitivity. Such differences include, but are not limited to, differences in excitation maxima, emission maxima, fluorescence lifetimes, fluorescence emission intensity at the same excitation wavelength or at a different wavelength, absorptivity, fluorescence polarization, or combinations thereof. Thus, the detectably different dye has different spectral properties and/or different selectivity.

In one aspect of the invention, the two or more dyes forming the dye-microparticle complex have the same or overlapping excitation spectra, but possess distinctly different emission spectra, generally having emission maxima separated by greater than 10 nm, preferably greater than 20 nm, and more preferably greater than 50 nm. Simultaneous excitation of all fluorescent reagents may require excitation of the sample at a wavelength that is suboptimal for each reagent individually, but optimal for the combination of reagents. Alternatively, the additional reagent(s) can be simultaneously or sequentially excited at a wavelength that is different from that used to excite the subject dye.

A preferred category of fluorescent dyes used in this invention are of the general class known as polymethine cyanine dyes, with typical emission wavelengths falling between 550 nm and 900 nm. Spectral properties, including emission wavelengths, are strongly influenced by the number of

methine groups. Thus, the monomethine dyes that are pyridines typically have blue to blue-green fluorescence emissions, whereas those that are quinolines typically have green to yellow-green fluorescence emissions. On the other hand, the fluorescence emissions of the trimethine dye analogs are substantially shifted toward red wavelengths, and those of pentamethine dyes are shifted even further, often exhibiting infrared fluorescence emissions (for example, see U. S. Patent No. 5,760,201). Although polymethine cyanine dyes constitute a preferred category of dyes for practice of the present invention, any dye that is soluble in an organic solvent (i.e., hydrophobic dyes) can be used when organic liquids are to be labeled.

On the other hand, when predominantly aqueous liquids are to be labeled, the above-indicated hydrophobic dyes, including the hydrophobic particles to which they are attached, may be used by subsequently or previously attaching hydrophilic groups to increase the solubility in aqueous solutions. In addition, ranges of hydrophilicity and hydrophobicity for liquids (i.e., different proportional mixtures thereof) are contemplated for mixed solvents, such as oil-in-water, water-in-oil, micelles, mixed micelles, detergent solutions, etc.

Squaric acid based fluorescent dyes can be synthesized by methods described in the literature; for example, see Sprenger, et al. *Angew. Chem.*, 79, 581 (1967); *Angew. Chem.*, 80, 541 (1968); and Maaks, et al. *Angew. Chem. Int'l. Ed.*, 5, 888 (1966). Briefly, one equivalent of squaric acid (1,2-dihydroxycyclobutenedione) is condensed with two equivalents of an active compound, such as a pyrrole, indoline, or aniline, and refluxed in a mixture of an alcohol and an aromatic solvent (such as benzene) under conditions that allow removal of water from the reaction mixture. The resulting dye can be collected and purified by any of such standard methods as recrystallization, distillation, chromatography, etc. Additionally, unsymmetrically substituted squaric acid compounds can be synthesized by methods such as those described by Law, et al., *J. Org. Chem.*, 57, 3278, (1992). Specific methods of making such dyes are well known in the art, and can be found, for example, in U.S. Patent Nos. 5,795,981; 5,656,750; 5,492,795; 4,677,045; 5,237,498; and 5,354,873. Some practical uses of such fluorescent dyes, for example, phthalocyanines, 2,3-naphthalocyanines, squaraines and croconic acid derivatives, are disclosed in U.S. Patent No. 5,525,516 to Krutak, et al.

Optionally, such dyes will contain functional groups capable of forming a stable fluorescent product with functional groups typically found in biomolecules or polymers including, for example, activated esters, isothiocyanates, amines, hydrazines, halides, acids, azides, maleimides, alcohols, acrylamides, haloacetamides, phenols, thiols, acids, aldehydes and ketones.

In addition to the specific squaric acid fluorescent dyes that are used in this preferred embodiment, related dyes can be further selected from such classes of compounds as cyclobutenedione derivatives, substituted cephalosporin compounds, fluorinated squaraine

compositions, symmetrical and unsymmetrical squaraines, alkylalkoxy squaraines, and squarylium compounds. Some of these dyes can fluoresce at near infrared as well as at infrared wavelengths -- effectively expanding the range of emission spectra up to about 1,000 nm. In addition to squaraines (i.e., derivatives of squaric acid), hydrophobic dyes, such as phthalocyanines and naphthalocyanines, also can be selected when operation at longer wavelengths is advantageous. Other classes of fluorochromes are equally suitable for use as dyes according to the present invention. Non-limiting examples of these dyes include 3-Hydroxypyrene 5,8,10-Tri Sulfonic acid, 5-Hydroxy Tryptamine (5-HT), Acid Fuchsin, Acridine Orange, Acridine Red, Acridine Yellow, Acriflavin, AFA (Acriflavin Feulgen SITSA), Alizarin Complexon, Alizarin Red, Allophycocyanin, ACMA, Aminoactinomycin D, Aminocoumarin, Anthroyl Stearate, Aryl- or Heteroaryl-substituted Polyolefin, Astrazon Brilliant Red 4G, Astrazon Orange R, Astrazon Red 6B, Astrazon Yellow 7 GLL, Atabrine, Auramine, Aurophosphine, Aurophosphine G, BAO 9 (Bisaminophenyloxadiazole), BCECF, Berberine Sulphate, Bisbenzamide, BOBO 1, Blancophor FFG Solution, Blancophor SV, Bodipy Fl, BOPRO 1, Brilliant Sulphoflavin FF, Calcien Blue, Calcium Green, Calcofluor RW Solution, Calcofluor White, Calcophor White ABT Solution, Calcophor White Standard Solution, Carbocyanine, Carbostyryl, Cascade Blue, Cascade Yellow, Catecholamine, Chinacrine, Coriphosphine O, Coumarin, Coumarin-Phalloidin, CY3.1 8, CY5.1 8, CY7, Dans (1-Dimethyl Amino Naphaline 5 Sulphonic Acid), Dansa (Diamino Naphthyl Sulphonic Acid), Dansyl NH-CH3, DAPI, Diamino Phenyl Oxydiazole (DAO), Dimethylamino-5-Sulphonic acid, Dipyrrometheneboron Difluoride, Diphenyl Brilliant Flavine 7GFF, Dopamine, Eosin, Erythrosin ITC, Ethidium Bromide, Euchrysin, FIF (Formaldehyde Induced Fluorescence), Flazo Orange, Fluo 3, Fluorescamine, Fura-2, Genacryl Brilliant Red B, Genacryl Brilliant Yellow 10GF, Genacryl Pink 3G, Genacryl Yellow 5GF, Gloxalic Acid, Granular Blue, Haematoporphyrin, Hoechst 33258, Indo-1, Intrawhite Cf Liquid, Leucophor PAF, Leucophor SF, Leucophor WS, Lissamine Rhodamine B200 (RD200), Lucifer Yellow CH, Lucifer Yellow VS, Magdala Red, Marina Blue, Maxilon Brilliant Flavin 10 GFF, Maxilon Brilliant Flavin 8 GFF, MPS (Methyl Green Pyronine Stilbene), Mithramycin, NBD Amine, Nile Red, Nitrobenzoxadidole, Noradrenaline, Nuclear Fast Red, Nuclear Yellow, Nylosan Brilliant Flavin E8G, Oregon Green, Oxazine, Oxazole, Oxadiazole, Pacific Blue, Pararosaniline (Feulgen), Phorwite AR Solution, Phorwite BKL, Phorwite Rev, Phorwite RPA, Phosphine 3R, Phthalocyanine, Phycoerythrin R, Polyazaindacene Pontochrome Blue Black, Porphyrin, Primuline, Procion Yellow, Propidium Iodide, Pyronine, Pyronine B, Pyrozal Brilliant Flavin 7GF, Quinacrine Mustard, Rhodamine 123, Rhodamine 5 GLD, Rhodamine 6G, Rhodamine B, Rhodamine B 200, Rhodamine B Extra, Rhodamine BB, Rhodamine BG, Rhodamine WT, Rose Bengal, Serotonin, Sevron Brilliant Red 2B, Sevron Brilliant Red 4G, Sevron Brilliant Red B, Sevron Orange, Sevron Yellow L, SITS

(Primuline), SITS (Stilbene Isothiosulphonic acid), Stilbene, Snarf 1, sulphO Rhodamine B Can C, Sulpho Rhodamine G Extra, Tetracycline, Texas Red, Thiazine Red R, Thioflavin S, Thioflavin TCN, Thioflavin 5, Thiolyte, Thiozol Orange, Tinopol CBS, TOTO 1, TOTO 3, True Blue, Ultralite, Uranine B, Uvitex SFC, Xylene Orange, XRITC, YO PRO 1, or combinations thereof. One skilled in the art will readily know which one to select among such dyes, in part based on the emission, absorption, and hydrophobic/hydrophilic properties required for a particular application. The spectral properties of the fluorescent dyes often will be sufficiently similar in excitation wavelengths and intensity to fluorescein or rhodamine derivatives to permit the use of the same flow cytometry equipment. However, generally it is preferable that a dye have high solubility in the particular liquid that is to be "marked," and improved photostability and quantum yields also are advantageous.

More preferably, the multiple dyes used in a single application or in a related series of applications will have the same or overlapping excitation spectra, but possess distinguishable emission spectra ("spectrally distinct"). Any detection system can be used to detect differences in spectral characteristics between two or more dyes, including a solid state detector, photomultiplier tube, photographic film, or eye -- any of which can be used in conjunction with additional instrumentation or methodology, such as a spectrometer, luminometer microscope, plate reader, fluorescence scanner, flow cytometer, confocal microscope, scanning microscope, epifluoresis detector, digital (CCD) camera, video camera, photographic film, visual inspection, photodiode, quantum counter, photomultiplier tube, capillary electrophoresis detector, or any combination thereof, to complete a detection system. Preferably, dyes will have emission maxima separated by greater than 10 nm, more preferably greater than 25 nm, and even more preferably greater than 50 nm. When differentiation between two or more dyes is accomplished by visual inspection, the two or more dyes preferably have emission wavelengths of perceptibly different colors to enhance visual discrimination. When it is desirable to differentiate between two or more dyes using instrumental methods, a variety of filters and diffraction gratings are commercially available to allow the respective emission maxima to be independently detected. When two or more dyes are selected that possess relatively small differences in emission maxima, instrumental discrimination can be enhanced by ensuring that the emission spectra of the two or more dyes have similar integrated amplitudes and similar emission peak widths, and that the instrumental system's optical throughput will be equivalent across the emission peak widths of the respective two dyes. Instrumental discrimination can also be enhanced by selecting dyes with narrow emission peak widths; however, such dyes must necessarily possess high amplitude emissions, or be present in sufficient concentration that the loss of integrated signal strength is not detrimental to signal detection/discrimination, for example, as measured by the signal-to-noise ratio.

6.3 Staining Process

The present invention describes techniques for precisely dyeing particles of varying sizes. The size of particles is immaterial to this invention since the precision of the dyeing process is not affected, except that (1) a given set of particles must be of uniform size within tight tolerances, for example, about ±10%, more preferably about ±5%, even more preferably about ±3%, and most preferably about ±2%, and (2) a particular application often will require a specific particle size, or range of sizes or size tolerance. Typically, particles are made of water-insoluble material, but may be made miscible or soluble in aqueous solvents by the addition of groups to increase hydrophilicity. The dyes employed are preferably squaric acid-based molecules that exhibit fluorescence extending into near infrared and/or infrared region, i.e., to about 1,000 nm. Use of other dyes may allow one to expand the range from the infrared to ultraviolet. The present method allows for a highly reproducible process in which two or more dyes of independent concentration are absorbed uniformly into or onto each particle, resulting in multiple spectrally distinct fluorescent signals corresponding to the number of different dyes present in or on the particle.

The following Examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These Examples are not intended in any way to limit the scope of the disclosure or the protection granted by Letters Patent hereon.

6.3.1 Example 1: General Outline of the Fluorescent Staining of Particles

A 5-10 gram (dry weight) quantity of stock particles in an aqueous medium is pipetted onto a vacuum filter membrane, and the liquid is removed and discarded. Next, a quantity of the rinse solvent (for example, 100 ml of an aliphatic alcohol, such as propanol, methanol, ethanol, etc.) is added to the particles. The particles are resuspended by placing an ultrasonic probe directly into the solution and applying power for several seconds, or as needed to effect resuspension. The suspension then is filtered, and the previous step is repeated once more. Dyeing of the particles is accomplished by adding 50 ml of a dye solution (composed of one or more dyes in an organic solvent) to the filtering cup and resuspending as before. The suspension then is allowed to sit for five minutes in the filtering cup. Next, 50 ml of rinse solvent is added to the dye suspension, sonicated and filtered. Another 100 ml of the rinse solvent is added, resuspended, and filtered. The last step is repeated once more. In order to prepare the particles for storage, 100 ml of an aqueous medium is added to the particles, the solution of which is then sonicated and filtered. Finally, 50 ml of aqueous medium is added to the particles, followed by sonication and transfer to a storage container.

In a particular embodiment of the invention, one or more dyes are mixed in a solvent suitable for the complete dissolution of the dyes, such as chloroform. Ethanol is added to the solution to increase wetting of the particles, and to create a process-dependent, final solvent density that is less than that of the particles. The concentration of each of the one or more dyes is experimentally determined as a function of the target fluorescence intensity at each of two center wavelengths of two fluorescence channels of the measuring instrument. These concentrations maintain their relative intensity throughout this inventive process.

An important aspect of the present invention is the preparation of particles prior to the dyeing operation. Manufacturers often supply particles in an aqueous medium. It has been discovered that the surfaces of particles that have been stored in an aqueous medium must be treated to make them permeable to organic compounds. In a preferred embodiment, a quantity of a polar organic solvent, such as an alcohol, is added to the particle solution to achieve about a 50-50 mixture of the aqueous medium and the polar organic solvent. This ratio, however, can be varied and adjusted at will according to particular needs that one may have, or determined by chemical and physical properties of the medium and the solvent.

An equally efficient and precise alternate technique entails "drying" the particles through a series of alcohol rinses, e.g., with methanol, ethanol, 2-propanol, rinses. The process is begun by spinning down the aqueous suspension of particles, typically about 10% solids (v/v) in suspension. The aqueous medium is decanted, and the particles are re-suspended in methanol. The alcohol solution with about 5% solids is vortexed, sonicated, and spun down. This step is performed once or twice more. Excess alcohol is decanted from the pellet, and residual solvent is evaporated under vacuum.

Test samples consisting of 0.05 gram quantities of dried particles are used to help adjust the dye solution to its desired ratio. Each dried 0.05 gram quantity of particles is suspended in 0.5 ml of a dye mixture containing two or more dyes of interest. The suspension of particles, now at 10% solids (w/v), is vortexed, and sonicated into suspension. Once in suspension, the mixture of particles and dyes is mixed for one hour. After that hour, the particles are spun down for a period of one minute using a centrifuge. The dye solution is then decanted, and the 0.05 g quantity of particles is re-suspended in 1 ml of 90% alcohol, e.g., methanol. The rinse step uses double the volume of the dye solution, thus maintaining a 5% solid solution. The sample is vortexed, sonicated, and spun down. The methanol supernatant is decanted. The 90% methanol rinse step is repeated once more. Finally, the excess methanol is decanted from the pellet, and the particles are re-suspended in an aqueous medium. Each test sample is then tested to determine the fluorescence activity of the labeled particles.

When the test samples show that the dye solution has the precise ratios of the desired dyes, a macro-scale batch is conducted. The principle of macro-scale work up is identical to that noted above. Briefly, (1) 25 ml of the desired dye solution is transferred to a 50 ml vial, which contains a 2.5 gram quantity of dried particles. (2) The particles, now at 10% solids (w/v), are vortexed and sonicated. (3) Once the particles are completely in suspension, the solution is mixed for an hour. After that hour, (4) the particles are separated from the dye solution by centrifugation, followed by decanting. (5) The 2.5 gram quantity of particles is then re-suspended in 50 ml of 90% methanol. The rinse step uses double the volume of the dye solution, thus maintaining a mixture of 5% solids. (6) The sample then is vortexed, sonicated, and spun down, and the methanol supernatant is decanted. (7) Step 6 is repeated once more. (8) After the final methanol rinse is decanted, the particles are put through an aqueous rinse. (9) The aqueous supernatant is decanted, and the beads are then re-suspended and stored in a fresh aqueous medium.

6.3.2 Example 2: Dyeing of 300 nm Amino Functionalized Polystyrene Particles

A 1% (w/w) organic solvent solution of particles (300 nm diameter polystyrene, amino functionalized) is stirred in a round bottom flask. To this is added a solution of the desired dye(s) in an organic solvent, such as chloroform. When the dye solution is no longer absorbed by the particles (determined empirically by prior experiments), addition of the dye is halted, and the solvent is quickly removed under reduced pressure. For some reaction conditions, the dyeing reaction can be halted within about ten minutes; however, this time depends upon a variety of reaction parameters. Alternative embodiments therefore include times shorter than and times longer than ten minutes.

6.3.3 Example 3: Use of Two (Red and Orange) Squaraine Dyes

In another embodiment, two fluorescent squaraine dyes are used, for example, a red fluorescent dye, such as 1,3-bis[(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)methyl]-2,4-dihydroxy-cyclobutenediylium, bis(inner salt), and an orange fluorescent dye, such as 2-(3,5-dimethylpyrrol-2-yl)-4-(3,5-dimethyl-2H-pyrrol-2-ylidene)-3-hydroxy-2-cyclobuten-1-one. These dyes are used to stain two separate populations of particles. Preferably, both dyes are excitable at approximately the same absorption wavelength, for example, ranging from an ultraviolet wavelength to about 800 nm, and emit fluorescent light at two distinct, essentially non-overlapping wavelengths that are separated from each other by at least 10 nm, preferably by at least 30 nm, and more preferably by at least 50 nm. For example, the emission peak of the first dye above is 585 nm, and the emission peak of the second dye is 630 nm. These dyes are chosen because they fall in the center of two of the fluorescence channels of a Becton Dickinson FACScan flow cytometer, which is

the measurement device used in a preferred embodiment of the present invention. However, the choice of fluorescence channels is relative and immaterial since different flow cytometry apparatuses can have different settings.

Once these dyes are embedded into or onto a particle, the total dye quantity will be between about 0.00001% and about 15% (w/w) of the final, total particle weight. However, this limitation is of little consequence to the present invention as long as the particles impregnated with said dyes are stable and usable for the intended purpose(s).

Optimal staining with a particular dye is dependent upon the physical and chemical parameters of the individual dye(s), the polymeric substrate(s), and the dye medium or solvent. Incubation times can vary widely depending on the desired results, the concentration of the dye(s), the chemical compositions and physical properties of the particles, and the reaction conditions. The optimal time is usually the minimum time required for the dye, in the concentration being used, to achieve the highest specific signal, while avoiding degradation of the dye and/or the particle over time, and minimizing all other undesirable fluorescent signals due to the dye(s).

In a preferred embodiment, chlorinated solvents such as chloroform are used to solubilize the dye(s). In alternative embodiments, suitable solvents are selected based on their ability to solubilize the particular class of hydrophobic dyes of interest. For example, the solvents can be acyl, aliphatic, cycloaliphatic, aromatic, or heterocyclic hydrocarbons. In further embodiments the solvents can have halogens, oxygen, sulfur, nitrogen, and/or phosphorus as either terminal groups or as internal/integral parts of a ring or chain. Specifically, solvents such as any of the alcohols, ethyl acetate, toluene, xylene, hexane, pentane, benzene, ether, acetone, various oils, carbon tetrachloride, carbon disulfide, DMSO, or methylene chloride can be used. In addition, other solvents known in the art can be used, such as the solvents listed in the *Merck Index* (Eleventh Edition; see sections MISC-63-68).

6.3.4 Example 4: General Procedure Used to Produce the X-Y Plot of Figure 1

A 5 ml quantity of undyed stock particles is placed in an aqueous medium directly on a membrane covered fritted funnel. A vacuum pump pulls air through the particles plated onto the filter paper for about one hour. Next, the dried particles are transferred to 50 ml of dye solution (having at least two dyes with spectrally distinct emission spectra), covered, and stirred at room temperature for about an hour. The particles then are separated by filtration from the dye solution, and the dyed particles are placed in a vacuum dessicator for about four hours to remove residual solvent. Next, a 200 ml quantity of Triton X-100 and water is added to the dried dyed particles in a

250 ml flask. The solution then is stirred for about three hours, followed by filtering and washing, which are repeated until no further dye is detected in the filtrate.

This procedure prepares a particular population of particles whose fluorescence emission spectra fall within a prescribed region or cluster depicted in a Fluorescence Particle Map, such as one of the ovals of Fig. 1. Typically, about 80% or greater of the individual particles within a particular set exhibit fluorescence characteristics within the desired region, preferably about 90% or greater, more preferably about 97% or greater, and most preferably about 99% or greater. When dyeing conditions are tightly controlled, a particular set of particles typically will exhibit about 1% or less of the individual particles having fluorescence characteristics that fall outside the desired region ("dispersion"), preferably about 0.5% or less, more preferably about 0.3% or less, and most preferably about 0.2% or less.

As an example, Figure 1 illustrates a 64-set particle collection, wherein each population or set differs from another by a distinct location or cluster on the X-Y plot. These locations essentially do not overlap. As opposed to the prior art methods that typically result in up to 10-20% or even higher rates of dispersion, the instant method allows production of essentially homogeneous populations or sets of particles having only about a 0.2 -0.3 % dispersion, which constitutes a large and significant improvement over the prior art.

Where nanoparticles have been dyed, the resulting dyed nanoparticles can then be linked to microparticles by any of the various well-known coupling reactions, such as carbodiimide coupling. Other methods of coupling can use such groups as carboxylates, esters, alcohols, carbamides, aldehydes, amines, sulfur oxides, nitrogen oxides, or halides.

6.4 Discriminators and Discrimination Power

Figure 1 illustrates eight levels of each of two dyes that are attached to particles according to practice of the present invention, which permits the creation of an 8 x 8 = 64 array of categories of fluorescently distinguishable particle sets. Fluorescently distinguishable discriminators thus provide the basis for creating arrays of categories of particle sets.

Additional discriminators operate similarly. For example, addition of a third dye at eight different levels requires 3-dimensional representation of an 8 x 8 x 8 = 512 array of categories of fluorescently distinguishable particle sets.

Other practical discriminators include size of particle, shape of particle, magnetic particle or particle with other magnetic property, dye type, radioactive property, chemical reactive group, nuclear magnetic resonance, dye fluorescence absorption, dye fluorescence emission , dye

absorption, fluorescence polarization, fluorescence lifetime, electron spin resonance, positron emission, concentration of particles, particle hydrophobicity, particle hydrophilicity, and particle density. For example, if two sizes of particles and two categories of magnetism (with or without magnetism) are added to the above 3-dimensional array, the array becomes 8 x 8 x 8 x 2 x 2 = 2,048 categories of particle subsets. Sizes and shapes of particles can be determined by light scattering measurements of forward and side scatter. The size of the particle can be measured in most flow cytometry apparatuses by forward or small-angle scatter light. Particle sets can also be further distinguished by differentiating between shapes of the particles, for example, using a high-resolution slit-scanning method. Magnetic particles incorporate magnetic or magnetically responsive metal oxides (for example, superparamagnetic, paramagnetic, or ferromagnetic oxides) into the polymer or polymer matrix.

Another category of discriminators is that of reactive groups that are attached to the particles, where each reactive group is capable of reaction with a specific compound. In some cases, the use of such discriminators will require two or more additional steps (for example, addition of a compound, followed by an incubation period, and measurement of an electromagnetic parameter, such as absorption, fluorescence emission, or magnetic resonance) to determine the reactive group and, hence, the category of the particle. In some cases, chromophores can be attached to the particles so that just a single optical or other measurement is required to determine the presence or absence of the chromophore on a given particle. Such discriminators provide powerful additional discrimination power. For example, as but one conservative calculation, use of 20 such discriminators in a labeling operation increases the above discussed array to 8 x 8 x 8 x 2 x 2 x 20 = 40,960 categories of particle sets.

An additional embodiment is that of inclusion of quantum dots within, on, or attached to particles. Quantum dots are fluorescing crystals which are excitable by a large range of wavelengths of light, but which have emission wavelengths that vary according to the size of the crystal -- with short wavelengths of emission occurring with small particles, and long wavelengths occurring with large particles. Thus, a large range of sizes of the crystals is used to create a large number of discrete emission wavelengths, creating a powerful expansion of discrimination power. Many dozens of classes of particles therefore are created according to the number of size classes of the quantum dot crystals. The size classes of the crystals are created either 1) by tight control of crystal formation parameters to create each desired size class of particle, or 2) by creation of batches of crystals under loosely controlled crystal formation parameters, followed by sorting according to desired size and/or emission wavelengths. Use of quantum dots for labeling particles, in the context of the present invention, is new, but is old in the art of semiconductors. Two examples of earlier references in

which quantum dots are embedded within intrinsic silicon epitaxial layers of semiconductor light emitting/detecting devices are United States Patent Nos. 5,293,050 and 5,354,707 to Chapple-Sokol, et al.

6.5 Methods of Labeling Liquids and Uses Therefor

The present invention basically entails the addition of particles, such as core microparticles and/or carrier microparticles having attached nanoparticles, to liquids as a labeling means.

Once a plurality of particle sets is produced, labeling a liquid simply requires adding the correct category of particle set(s) to the liquid that is to be so labeled according to that particular category of particle set(s). In a preferred embodiment, the particles are impregnated with three different fluorescent dyes in eight different concentrations or levels of each of the dyes so as to create 512 subsets of particles. Each particle is later classified into its member subset using a measurement instrument. These 512 subsets of particles comprise 512 labeling elements or discriminators. Depending on the needs of the user, these discriminators can be combined with other discriminators to increase the number of unique labels, as discussed in section 6.4. For example, the presence or absence of a labeling element yields certain permutations. Using eight levels of a discriminator at a time, the presence or absence of a discriminator gives (512!/8!(512-8)!), or approximately 10^{17} possible unique labels. In addition, varying the concentration of one or more uniquely labeled particle subsets expands the possibilities further, particularly when a uniform distribution of particles is achieved in the liquid. Also, a check digit can be added which has half or other fraction of the number of particles as the identifying particles.

In one embodiment, a signature can be created to meet the needs of a particular application by using unique particle sets. For example, a donor "Social Security Number" can be created by 1) assigning nine different order discriminators to nine different particle sets, where each particle set is respectively assigned to one of the nine ordered digit positions that make up a Social Security Number (i.e., ones, tens, hundreds, thousands, . . . to hundred millions), and 2) further categorizing each of the order class particle sets into one of ten numerical classes of digits zero through nine by assigning each of ten different levels of each of the nine order class discriminators to correspond to a selected digit that is different from the remaining nine digits. For example, order discriminators are assigned based on relative ratios of red to orange fluorescing dyes in or on particles as follows. A digit in the one's position is encoded by a first relative concentration ratio of red to orange fluorescing dyes, a digit in the ten's position is encoded by a second relative concentration ratio of red to orange fluorescing dyes, a digit in the hundred's position is encoded by a third relative concentration ratio of red to orange fluorescing dyes, etc. In a first embodiment, specific digits zero

through nine (i.e., the ten numerical classes) are then encoded according to the absolute amounts of the two dyes ("levels of the two dyes") in or on each particle. Alternatively, in a second embodiment, ten different concentrations of the order discrimination particles (or ten different total numbers of particles/sample) may be used to represent the ten digits. In this way, a single discriminator can serve the dual role of providing discrimination with respect to two different encoding aspects. In particular, in the second embodiment, only nine sets of beads are needed to encode any Social Security Number. Of course, other discriminators can be used with equal efficiency and practicality. Obviously, variations in level of a discriminator need only be such as to permit ready discrimination by the instrumentation to be used to read the Social Security Number. Furthermore, the progression of the ten digits of the ten numerical classes need not be represented by a monotonically increasing level of a discriminator(s) for the progression; rather, a monotonically decreasing level of the discriminator(s) may be employed, or any other pattern that achieves the goal of unique classification of each of the ten numerical classes. Also, separate order and numerical discriminators can be used (a 10 x 9 = 90 array of particles); however, use of such an array to encode a Social Security Number(s) usually is not as simple and not as economical as an encoding scheme that utilizes just one category of discriminator, or a combination of discriminators, to define the nine ordered digit positions, with attendant encoding of digits zero through nine by use of levels of the selected discriminator(s). However, a second discriminator (for example, particle density) in some applications may be used advantageously to provide numerical class encoding, for example, ten different densities of particles to represent digits zero through nine within each of the nine order class particle sets; alternatively, a range of nine different particle densities can serve as the nine order discriminators. In such an application, particles with the lowest density usually will be required to have a density greater than the liquid(s) they encode.

Analogous to the coding for a patient's Social Security Number, a similar scheme can be used to encode desired alphanumeric information of a patient, such as birth date, date of sample collection, name of patient, etc. To provide the most parsimonious and efficient use of particles, order discriminators are utilized according to the number of desired alphanumeric characters for the encoding. Then, as in one encoding embodiment for encoding a Social Security Number, concentrations of particles and/or other discriminators are used. For example, if 20 character positions are required for the encoding, and only alpha characters are to be used, 26 concentrations of particles would be required; alternatively, 10 concentrations of particles x 3 levels of a second disriminator to create 30 possible alpha characters for each of the 20 character positions; or any other such combination of discriminators to create the 26 required alpha characters. Also, characters such

as "space" and apostrophe, etc., can be incorporated. In addition, if liquid labeling identifies the patient sample, virtually all laboratory errors can be detected, including inter-sample contamination.

In another embodiment, a date code is created for labeling a liquid, for example, with respect to the date that a sample is taken from the liquid, an expiration date, a birth date, an expiration date for a perishable liquid, etc. For example, one of an array of 12 particle sets is assigned to the month January, a second to February, etc.; one of an array of 31 particle sets is assigned to the date 01, a second to 02, and so on through the date 31; and one of an array of five or more particle sets is assigned to a first year of interest, for example, 1999, a second to 2000, etc., for five consecutive years. This yields over 1800 exact dates by using only distinct 48 particle sets (more if greater than 5 years are used).

An alternative embodiment for encoding dates uses the concept of a series of small arrays. For example, four different "month" particles (having $2^4 = 16$ possible combinations) can be used to encode the 12 months, leaving 4 unused combinations; five different "day" particles are then assigned to encode the 31 days ($2^5 = 32$ possible combinations, leaving one unused combination); and five different "year" particles are then used to encode 32 years. Thus, only 14 different particles need be used to encode all the days over a span of 32 years. Furthermore, use of the unused combinations would further expand this span of years. For example, simple direct use (i.e., one particle combination per year) of the five unused particle combinations could be employed to add another 5 years of encoding coverage. Alternatively, use of the 4 unused month particle combinations, together with the presence or absence of the one unused day particle, would permit encoding of an additional 8 years, or 40 years total.

The concept of use of a series of small arrays of different particles can readily be applied to other encoding situations, including for routing, alphanumeric representation, Social Security Number, etc. In addition, the concept of "present" versus "absent" for different particles, that underlies calculation of numbers of possible permutations, may be applied in the form of "high" and "low," where high and low refer, for example, to high and low concentrations of particles in a sample, to high and low absolute numbers of particles in a sample, to high and low concentrations or levels of fluorescent dyes in or on the particles, to high and low levels of any other discriminator, etc.

Another embodiment for liquid labeling of the present invention is that of labeling solids, which later are solubilized, liquified, or extracted to recover particles contained therein for purposes of encoding. As but one illustrative example, microparticles are incorporated into sodium chloride crystals for such purposes as identification of manufacturer, lot number, production date, expiration date, degree of purity, etc. Such uses also include identification and/or tracing of pirated materials and products. Applications wherein solids are benficially labeled with microparticles are essentially

limitless, examples including glues and adhesives (for example, use on postage stamps, envelopes, memo pages that adhere to a wall, page, or other surface, etc.), ink, paint, explosives, plastics and plastic products, explosives, chemicals in general where minute quantities of an appropriate kind of microparticles do not interfere with the end use of the chemical(s), tracing dissolution attributes and flow patterns *in vivo* of pharmaceutical tablets, etc.

In yet another embodiment, microparticles are used to label gases. Typically, such a use will require microparticles of low density, unless the gas or gases are flowing sufficiently (and possibly including sufficient turbulent flow) to maintain the microparticles in suspension therein. As with applications in solids, uses of microparticles in gases have an essentially unlimited range of applications, for example, including for purposes of tracing and/or identifying pirated gases, tracing the flow of gases in an animal, and encoding of manufacturer, lot number, production date, physical properties, degree of purity, etc.

In an embodiment for encoding to route liquids through a complex of pipelines having a plurality of branch points, different particle sets are created to uniquely represent each desired destination for a plurality of liquids coursing through the complex of pipelines, and particles from a particular particle set are added in an appropriate amount (determined by experimentation) to the liquid to be encoded by that particle set. Discriminators and physical properties are selected to be consonant with the physical properties of the liquid to which the particles are to be added. For example, hydrophobic particles typically will be used in hydrophobic liquids unless the hydrophobic liquids also contain detergents or amphiphiles sufficient to evenly disperse the particles in the liquid. Similarly, the particles typically are selected to have a greater density than the liquid to promote even dispersion. Selection of the final concentration of particles in the liquid that is so encoded will depend on such factors as cost/benefit, and sensitivity of the instrumentation to perform the analyses on the particles. This embodiment functions by providing analysis at an appropriate point in the pipeline to which the particles are first added, for example, at or near and upstream from a branchpoint or decision point in a pipeline. Switches and valves are then connected to the instrumentation, and control the final routing per the desired destination, based on the one or more discriminators of the particular particle set.

Similar protocols may also be used in a clinical setting to direct one or multiple body fluid samples from one or more patients to the appropriate instrument to perform the desired clinical chemistry (multiplexed clinical assays). In such a clinical chemical setting, a plurality of body fluid samples can include the addition of a quantity of particles into each body fluid sample to correspond to the desired clinical chemical analysis and/or machine. In some cases, for example, it is desirable to route a body fluid sample to a machine that performs a panel of analyses. Typically, a flow

cytometer will be in-line with the tubing carrying the body fluid samples to detect routing particles and to transmit data on same to the appropriate switch(es) and valve(s) to direct the body fluid samples to the desired analytical site. Flow cytometers are the preferred instrument for such use, though any instrument that is capable of detecting the particular discriminator may be used.

Historically, the use of precision dyed particles in multiplexed clinical assays is provided, for example, by WO Patent No. 97/14028 and U.S. Patent No. 5,736,330, disclosures of which are herein incorporated by reference in their respective entireties. When these earlier disclosures are combined with the current invention, it is possible to greatly improve quality assurance in the clinical laboratory. For example, by selecting a unique permutation of particle sets, and permanently assigning that permutation to a particular assay, a fail-safe assay is created. A particle set also can be coupled to an associated reagent in a GMP (General Manufacturing Practice) environment, and combined into a single reagent vial. In addition, current multiplexed diagnostic instruments can be programmed to recognize a specific permutation, look up the parameters of that assay in a database, perform that assay, and record the results, thus eliminating the possibility that incorrect reagents will be added to a patient sample.

In embodiments for tracing toxic substances in groundwater, selection of the kinds of particles and their respective concentrations will include consideration of such factors as density and hydrophobicity of the toxic liquid(s), and size of the faults and/or densities of the soil(s) connecting a point toxic liquid pollution source with one or more aquifers of interest, as well as prospective or known dilution factors from underground water sources and/or above ground water sources, for example, recent rain or generally high rain fall. In addition, the sensitivity of the instrumentation to be used to decode or read the particles taken from a site distant from the point pollution source must be considered.

In embodiments for tracing absorption patterns of fluids in plants and animals, generally smaller particles will be advantageous; however, a large variation in particle size will be required for a wide range of tissue types, body cavities, etc. Factors similar to those of the preceding examples also require consideration in plants and animals. For example, particles that would be used in studies relating to bile flow would benefit from amphiphilic particles due to the presence of amphiphilic bile salts in bile. Of course, instrumentation sensitivity and dilution during use in an experiment must also be considered.

In general, a label is constructed by selecting one or more unique particle sets according to the particular application, adding them to the liquid, and mixing. The concentration of the particle volume with respect to the liquid volume also is a variable that is application dependent, for example,

reflecting the priority of cost versus speed: as the concentration of particles is increased, so, too, is the cost increased, whereas the reading time typically will be decreased.

In general and ideally, the particles are inert in the liquid being labeled. Furthermore, criteria for matching particle types to liquids include such parameters as stability of the particle in the particular liquid, stability of the dye attachment to the particle, and non-interference of the particle(s) with the use for which the liquid is intended. Thus, hydrophobic particle types generally will be used only with hydrophobic liquids, unless the hydrophobic liquid also contains detergents, amphiphiles, and/or hydrophiles. Particles that are used in organic solvents require that the dye or other discriminator be encapsulated in, or matrix entrapped by, a solvent stable polymer, or covalently attached to the particle.

Kits can also be produced to allow a user to mix particles in predetermined proportions and thereby appropriately label a liquid according to user defined or elected parameters. For example, a kit can feature 512 distinct particle sets based on eight levels of each of three fluorescent dyes. Alternatively, or in addition, other discriminators can be featured to create the number of distinct sets of particles necessary for use in a particular liquid labeling application, which also will take into account such attributes as hydrophobicity and density of the liquid to be labeled, as well as parameters such as stability of the fluorescent dye, or stability of matrix entrapment of the fluorescent dye, in the particular liquid. Specifically, a kit features particle sets for each desired discriminator, and each particle set comprises particle subsets which represent an appropriate range of values for that particular discriminator so as to create a functional range of subsets thereof. Thus, the user simply dispenses an appropriate quantity of each of the one or more sets/subsets of particles into the liquid selected to be labeled by that particular set(s)/subset(s) of particles. Furthermore, multiple classifications can be applied concurrently, for example, including one or more sets of particles to encode the patient's name and/or Social Security Number, and a second one or more sets of particles to encode routing for test procedures, storage, etc.

Another embodiment that can be coupled to a kit entails the use of a microprocessor controlled automatic pipetting system. In this embodiment, the user types into the system, using a standard keyboard, the desired numbers or other indicators (for example, for routing, indication of test to be performed, birth date of patient, Social Security Number, etc.). The user similarly supplies the desired microparticle type that is to correspond to a selected indicator; i.e., consideration is given to the physical properties or other bases for best matching particles with the liquids at hand. A simple software program allows the user to input the desired discriminators and the alphanumeric or other symbols to which the discriminators respectively correspond. Such programs are well known in the art, and may, for example, include lookup tables for the correspondence between

discriminators and symbols. Output signals from the microprocessor then operate to control automatic pipetting of the selected microparticles into one or more liquid or gaseous samples that are positioned in receiving modes relative to the pipetting system. Indicators indicate the purpose(s) for which the liquid or gas samples are labeled with particles.

The discriminators are used to distinguish particles based on differences in particle concentration, particle physical property, dye type, dye concentration, spectral property, label type, and combinations thereof. These broad categories of discriminators can be further categorized into other discriminators as follows. The particle physical property can include such attributes as particle size, particle shape, particle hydrophobicity, particle hydrophilicity, particle density, and combinations thereof. The spectral property can include such attributes as dye fluorescence absorption, dye fluorescence emission, dye absorption, dye fluorescence polarization, dye fluorescence lifetime, and combinations thereof. Label type refers to physicalchemical properties of chemical groups added to particles, and can include such attributes as magnetic property, (chemical) reactive group, nuclear magnetic resonance, electron spin resonance, positron emission, radioactive property, fluorescence polarization, fluorescence lifetime, and combinations thereof. Dye type refers to the chemical composition and structure of fluorescent dye molecules. Dye concentration refers to the concentration of the dye(s) on or in a particle. Particle concentration refers to the concentration of particles in the liquid being labeled.

When a liquid is ready for analysis or decoding of its label, the liquid is thoroughly mixed, and a sample is withdrawn. Next, a centrifugation, filtering, magnetic, or other separation means is used to separate the particles from the liquid thereby so encoded. The particles are then analyzed.

When encoding is achieved by the use of a series of spectrally distinct dyes, various commercially available instruments can be used to read the liquid labels. For example, a fluorescence microscope can be used on a sample of the liquid to locate and characterize the particles in the sample. In a preferred embodiment, a flow cytometer is used according to procedures known in the art, such as is disclosed in U.S. Patent No. 4,665,024 to Mansour, et al. Flow cytometry is an optical technique that analyzes particles in a fluid mixture based on the particles' optical characteristics using a flow cytometer. Background information on flow cytometry is set forth in Shapiro, Practical Flow Cytometry, Third Ed. (Alan R. Liss, Inc. 1995), incorporated herein by reference in its entirety. A flow cytometer can automatically display the clusters formed by the fluorescent emissions of the particle sets, for example, as shown herein in Fig. 1. In a preferred embodiment, the host computer that controls the flow cytometer is programmed to automatically detect the clusters and convert the particle set information into its ordinal information, such as date, "Social Security Number," patient's name, patient's birth date, or other identifying parameter.

Other means for detection and analysis can also be utilized, including, but not limited to, visual inspection, digital (CCD) cameras, video cameras, photographic film, or instrumentation such as laser scanning devices, fluorometers, photometers, luminometers, photodiodes, quantum counters, plate readers, epifluorescence microscopes, scanning microscopes, confocal microscopes, capillary electrophoresis detectors, or any other means that provides amplification of an electromagnetic signal, such as a photomultiplier tube or other light detector capable of detecting the presence, location, and intensity of excitation and emission spectra, fluorescence polarization, fluorescence lifetime, and other physical properties of a fluorescent or other electromagnetic signal.

When particles are processed by a flow cytometer, measurement of four exemplary classification parameters [forward light scatter, side light scatter, a first fluorescence emission peak (for example, red fluorescence), and a second fluorescence emission peak (for example, orange fluorescence)] can be used to identify the set, population, or cluster to which each particle belongs. Furthermore, additional chromophores, reactive groups, and other discriminators can be measured. Although methods and products of the present invention have been described in detail for purposes of general illustration, it is understood that such detail is solely for that purpose, and that variations can be made therein and thereto by those skilled in the art without departing from the scope of the invention, which is defined by the following claims. For example, the use of sets of small arrays of particles to encode respective portions of an alphanumeric string can be applied to virtually any encoding application.

WHAT IS CLAIMED IS:

1. A method of labeling a gaseous, liquid, or solid material to permit the subsequent identification of the labeled gaseous, liquid, or solid material, which comprises adding to a gaseous, liquid, or solid material, whose subsequent identification is desired, a sufficient quantity of particles belonging to one or more distinguishable sets of particles, to provide a labeled gaseous, liquid, or solid material that subsequently can be identified.

2. The method of claim 1 in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set.

3. The method of claim 2 in which a sufficient quantity of a combination of particles belonging to two or more distinguishable sets of particles is added to the gaseous, liquid, or solid material, whose subsequent identification is desired.

4. The method of claim 2 in which said one or more discriminators is selected from a physical, chemical, biological, magnetic, or spectral property of the particle or combinations thereof.

5. A method of identifying a gaseous, liquid, or solid material that has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles, which comprises:

(a) obtaining a sample comprising a gaseous, liquid, or solid material that has been labeled with a sufficient quantity of particles belonging to one or more distinguishable sets of particles;

(b) analyzing the particles found in the sample to determine which of one or more distinguishable sets of particles such particles belong to; and

(c) comparing the results of said analyzing step with a key which establishes the identity of the gaseous, liquid, or solid material from which the sample was taken.

6. A labeled material which comprises (i) a gas, liquid, solid, or combinations thereof and (ii) a sufficient quantity of particles that can be shown to belong to one or more distinguishable sets of particles.

7. The labeled material of claim 6 in which the particles in each set exhibit one or more discriminators that distinguish the particles of one set from those of another set.

8. The method of claim 1 in which said material comprises a gas.

9. The method of claim 1 in which said material comprises a liquid.

10. The method of claim 1 in which said material comprises a solid.

11. The method of claim 5 in which said material comprises a gas.

12. The method of claim 5 in which said material comprises a liquid.

13. The method of claim 5 in which said material comprises a solid.

14. The labeled material of claim 6 which comprises a gas.

15. The labeled material of claim 6 which comprises a liquid.

16. The labeled material of claim 6 which comprises a solid.

17. A kit for labeling liquids, solids, and gases comprising particles from a plurality of distinct particle sets for labeling liquids, solids, and gases, and a dispenser.

AMENDED CLAIMS

[received by the International Bureau on 18 August 1999(18.08.99);
original claim 6 amended; remaining claims unchanged (1 page)]

6. A labeled material whose subsequent identification is desired, which comprises (i) a gas, liquid, solid, or combination thereof and (ii) a sufficient quantity of particles that can be shown to belong to one or more distinguishable sets of particles.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07993

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B32B 5/16
US CL :425/6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 264/ 5,6,7; 425/5,6; 428/402,403,407; 436/518,525,529,533

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSTIC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,723,218 A (HAUGLAND et al) 03 March 1998, see entire document.	1-17
Y	US 4,981,625 A (RHIM et al) 01 January 1991, column 3, line 63 - column 11, line 63.	1-17
A	US 5,736,330 A (FULTON) 07 April 1998, column 2 line 16 - column 7, line 12.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 MAY 1999

Date of mailing of the international search report

18 June 1999 (18.06.99)

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
D. Lawrence Tor
GAILENE R. GABEL
Telephone No. (703) 308-0196